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Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout

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Abstract

Rainbow trout, as well as many other species of fish, demonstrate the ability to survive starvation for long periods of time. During starvation, growth rate is decreased and muscle exhibits signs of wasting. However, upon resumption of feeding, accelerated growth is often observed. Alterations in muscle metabolism occur during feed restriction and refeeding, although the ways in which these alterations affect the molecular pathways that control muscle growth have not been fully determined. To analyze changes in muscle metabolism and growth during starvation and refeeding, real-time PCR was used to test the expression of six metabolic-related genes and eight muscle-specific genes in rainbow trout white muscle prior to and after 30 days of starvation, and after 4 and 14 days of refeeding. The six metabolic-related genes chosen are indicative of specific metabolic pathways: glycolysis, glycogenesis, gluconeogenesis, the pentose phosphate pathway, and fatty acid formation. The eight muscle specific genes chosen are key components in muscle growth and structural integrity, i.e., *MRF*s, *MEF*s, *myostatin*s, and *myosin*. Alterations in expression of the tested metabolic-related genes and muscle-specific genes suggest that during both starvation and refeeding, changes in specific metabolic pathways initiate shifts in muscle that result mainly in the modification of myotube hypertrophy. The expression levels of many of the metabolic-related genes were altered during the refeeding period compared to those observed before the starvation period began. However, the accelerated growth often observed during refeeding is likely driven by changes in normal muscle metabolism, and the altered expression observed here may be a demonstration of those changes.
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1. Introduction

Methods for maximizing growth have been tested for many years in aquaculture. One of these methods, feed restriction and refeeding, results in varied levels of increased growth rate. Following nutritional restriction, many organisms attempt to make up for the growth that was lost by accelerating growth (Hornick et al., 2000). In rare cases, fish displaying this characteristic are able to surpass in size those individuals that did not undergo nutritional restriction (Nikki et al., 2004; Hayward et al., 1997), making the method of cyclic feed restriction and refeeding a possible tool for aquaculturists to maximize growth.

There are several hypotheses that attempt to explain the increased growth rate that normally ensues following a starvation period. One is that there is an increase in feed intake following starvation, driving the growth rate higher (Hayward et al., 1997), and another is that the hormonal response during and following starvation drives growth (Gaylord et al., 2001). Alterations in metabolism induced by starvation and refeeding result in changes in growth rate and concomitant body remodeling. Muscle is one of the tissues that is considerably affected by starvation and refeeding (Hornick et al., 2000). In fish muscle, glycogen is a short-term reserve, so during periods of starvation, lipid stores and muscle protein breakdown provide the bulk of energy (Mommsen and Moon, 2001). This results in a reduction in, and sometimes a cessation of, muscle growth. During refeeding, protein synthesis, first in the viscera and then in muscle, is accelerated relative to the rate of protein degradation (Hornick et al., 2000), restoring muscle

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growth. Since metabolic state largely determines the extent of muscle growth, it is important to understand whether metabolic changes are associated with changes in expression of genes known to influence muscle growth, and the ways in which this regulation may occur. To characterize the molecular effect on muscle of feed restriction followed by refeeding, the expression of six metabolic-related genes and eight muscle-specific genes was tested during starvation and refeeding.

The six metabolic-related genes tested were *phosphoglycerate kinase*, *pyruvate kinase*, *phosphoglucomutase*, *fructose 1,6-bisphosphatase*, *transaldolase*, and *aldolaseB*. These were chosen because, depending on nutritional state, they are indicative of the activation of the following specific metabolic pathways: glycolysis, glycogenesis, gluconeogenesis, the pentose phosphate pathway, and fatty acid synthesis. Both phosphoglycerate kinase and pyruvate kinase are important enzymes in glycolysis, the conversion of glucose into pyruvate with the concomitant production of ATP (Salway, 2004; Muñoz and Ponce, 2003). Phosphoglucomutase is an important enzyme in the reciprocal glycogen metabolism pathways of glycogenesis and glycogenolysis. It catalyzes the conversion of glucose-6-phosphate to glucose-1-phosphate in glycogenesis and the inverse in glycogenolysis (Levin et al., 1999). Gluconeogenesis, the production of glucose from glycerol, lactate, and amino acids, is dependent on fructose 1,6-bisphosphatase, which catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate (Tillmann et al., 2002). The pentose phosphate pathway, a mechanism of generating NADPH for fatty acid synthesis or ribose-5-phosphate for nucleotide and nucleic acid synthesis, is dependent on the enzyme transaldolase (Stryer, 1995). AldolaseB converts fructose 1-phosphate into dihydroxyacetone phosphate and glyceraldehyde, precursors to glycolysis, gluconeogenesis, and fatty acid formation (Shiokawa et al., 2002; Llewellyn et al., 1998).

Since specific aspects of muscle growth and breakdown are dependent upon nutritional status, genes involved in the myogenic signaling pathway were analyzed to determine the expression response to starvation and refeeding. Extensive research has delineated a molecular signaling pathway that controls vertebrate muscle growth. The primary MRFs (Muscle Regulatory Factors), MyoD and Myf5, specify myoblasts and control their proliferation, while the secondary MRFs, myogenin and MRF4, mediate differentiation and fusion of myotubes, forming myofibers, the functional unit of muscle (Parker et al., 2003). Other genes, such as the MEF2s and the myostatins, also influence this pathway, both positively and negatively. The MEF2s are thought to control mainly differentiation (Black and Olson, 1998), while the myostatins are negative regulators of both specification and differentiation (Thomas et al., 2000; Langley et al., 2002). In this study, the eight muscle-specific genes tested, *TMyoD2*, *TMyf5*, *Tmyogenin*, *MEF2A*, *MEF2C*, *myosin*, *Tmyostatin1* and *Tmyostatin2*, were chosen because they are the rainbow trout homologs of the mammalian genes involved in myogenic signaling (Rescan and Gauvry, 1996; Johansen and Overturf, 2005a,b; Rescan et al., 1995, 2001; Gauvry and Fauconneau, 1996). In many species, postnatal muscle growth occurs mainly by myotube hypertrophy, however in fish, postnatal muscle growth occurs by both hyperplasia (cell proliferation resulting in the generation of new myotubes) and hypertrophy (increase in myotube size) (Rowlerson and Veggetti, 2001), offering a post-embryonic system in which to study the regulation of these processes.

2. Materials and methods

2.1. Fish rearing conditions and experimental design

All fish rearing was carried out at the University of Idaho's Hagerman Fish Culture Experiment Station. The experiment was carried out in 140 L tanks with a water flow of approximately 11.5 L/min, and photoperiod was maintained at a constant 14 h/day. Rainbow trout of the CSI-Oregon strain, approximately 300 g, were used in the experiment. The fish had been fed to satiation with a commercial diet until the beginning of the experiment, at which time white muscle tissue was collected from 10 fish. The fish were then starved for 30 days, at which time white muscle tissue was collected from 10 fish. Feeding to satiation with a commercial diet was resumed for 14 days. At both four and 14 days of refeeding, white muscle tissue was collected from 10 fish. Fish were handled and treated according to the guidelines of the University of Idaho's Animal Care and Use Committee.

2.2. Sample collection and total RNA isolation

Tissue was collected and total RNA isolated at the following time points: day 0 (before starvation period), 30 days after beginning starvation, 4 days after refeeding (to satiation), and 14 days after refeeding (to satiation). RNA was isolated from white muscle; for all time points, $n=10$. All total RNA isolations were carried out using TRIzol according to the manufacturer's protocol (Invitrogen, Rockville, MD).

2.3. Quantitative real-time RT-PCR

To detect the level of gene expression at each time point, real time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA). The final concentration of each reaction was: Master Mix, 1× (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/μl; RNase inhibitor mix, 0.4 U/μl; forward primer, 900 nM; reverse primer, 900 nM; probe, 250 nM; total RNA, 50 ng. For *pyruvate kinase*, *myogenin*, *myosin*, *Tmyostatin1*, *Tmyostatin2*, and *β-actin*, primers and probe were designed using Primer Express software (ABI). For *phosphoglycerate kinase*, *phosphoglucomutase*, *fructose 1,6-bisphosphatase*, *aldolaseB*, *transaldolase*, *TMyoD2*, *TMyf5*, *MEF2C*, and *MEF2A*, primers and probe were designed by ABI's Assay by Design service. All primer and probe sequences are listed in Table 1. Cycling conditions for all genes tested except *pyruvate kinase*, *myosin*, and *β-actin* were as follows: 30 min at 48 °C,

Table 1
Genbank accession number and real-time quantitative RT-PCR primer and probe sequences for each tested gene

Gene	Genbank accession no.	Primer/probe sequence (listed 5′–3′)
Phosphoglycerate kinase	CX149068	PhoglyckinF: AGTGCCTAAGCCCCATCAC
		PhoglyckinR: GGGTGGGAGACAGAGCTTTT
		Phoglyckin-MGB: 6FAM-CCCGCCAGAGTTTG-NFQ
Pyruvate kinase	AF246146	PK-97F: TGCTGGAGCTGTCATAGTACTCACTA
		PK-182R: GCCACCTGAGGACTTCTTGTTACC
		PK-164T: 6FAM-CGCTGTCCCATCGTCGCG-TAMRA
Phosphogluco-mutase	BX074051	PhoglumutF: CGGGCCCCACTGTGATC
		PhoglumutR: GACGGTTGAAAGGACTGAGGTA
		Phoglumut-MGB: 6FAM-CTCTGCCACACCCTTC-NFQ
Fructose 1,6-bisphosphatase	AY113693	FR16bPaseF: CGTTATGTCGGCTCCATGGT
		FR16bPaseR: TGCCTCCGTACACCAGAGT
		FR16bPase-MGB: 6FAM-CCTGTGCACATCAGCC-MGB
Transaldolase	CA341774	TransaldF: TGGAGCAGCTGAAGAAACACA
		TransaldR: GGTTGGTGGTGGCATCCT
		Transald-MGB: 6FAM-CCGCCACGACCACCG-MGB
AldolaseB	CR366391	AldolaseBF: ACGCCAGTATCTGCCAACAG
		AldolaseBR: GTCTCCGTCAGGCAAAATCTCT
		AldolaseB-MGB: 6FAM-ATGGGCACCAGGCCGT-MGB
TMyoD2	Z46924	MyoD-AbD-F: GCCGTCACCGACCAACT
		MyoD-AbD-R: CACTGTGTTCATAGCACTTGGTAGA
		MyoD-AbD-MGB: 6FAM-CCGTCCCATGACCCC-NFQ
TMyf5	AY751283	Myf5-AbD-F: CACAAGCTATGGCAACAACTACAG
		Myf5-AbD-R: GGCACCAGCACCTCTCT
		Myf5-AbD-MGB: 6FAM-TCCAGAGCTCACATTCT-NFQ
Tmyogenin	Z46912	Myogenin-328F: CATGGACCGGCGGAAAG
		Myogenin-400R: GGCCTCGAATGCCTCGT
		Myogenin-377MGB: 6FAM-CTTCTTCAGCCTCCTCTT-NFQ
myosin	Z48794	Myosin-213F: TGCTGAACCTTCCTGAACTTAGA
		Myosin-287R: GGCCTACAAGAGGCATTCTG
		Myosin-242T: CTGGTTTGCTGCTTCCTCCGCTT-TAMRA
MEF2C	CA380324	MEF2C-AbD-F: CCCTAGGCAACCACAACCT
		MEF2C-AbD-R: ACTGGGAGGTCTATGTGTGACA
		MEF2C-AbD-MGB: 6FAM-CCGTCCCATGACCCC-NFQ
MEF2A	CA374878	MEF2A-AbD-F: GGCCAGGCAGCTCTCA
		MEF2A-AbD-R: TTGGAGCCCTGAGGTAGGT
		MEF2A-AbD-MGB: 6FAM-CACCCACCAAAGAGC-NFQ
Tmyostatin1	AF273035	Myostatin1-47F: CCGCCTTCACATATGCCAA
		Myostatin1-138R: CAGAACCTGCGTCAGATGCA
		Myostatin1-68MGB: 6FAM-CATATTACATTTGGGATTCAA-NFQ
Tmyostatin2	AF273036	Myostatin2-44F: AGTCCGCCTTCACGCAAA
		Myostatin2-164R: ACCGAAAGCAACCATAAAACTCA
		Myostatin2-64MGB: 6FAM-CGTATTCACTTTTGGATTTT-NFQ
β-actin	AF254414	β-actin-372F: TGGCCGTACCACCGGTAT
		β-actin-451R: GCAGAGCGTAGTCCTCGTAGATG
		β-actin-399: 6FAM-CTCCGGTGACGGCGTGACCC-TAMRA

10 min at 95 °C, then 40 cycles of PCR consisting of 15 s at 95 °C followed by 1 min at 60 C. Cycling conditions for *pyruvate kinase*, *myosin*, and *β-actin* were: 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C, then 40 cycles of PCR consisting of 20 s at 95 °C followed by 1 min at 62 °C. For each group, assays were run in duplicate on RNA samples isolated from individual fish.

Absolute copy number of each mRNA sample tested was determined by including standards consisting of in vitro transcribed mRNAs specific for each gene tested, with each set of experimental samples that were analyzed by real-time quantitative RT-PCR. To make standards, the same primers designed for real-time amplification were used in RT-PCR of total RNA isolated from white muscle or liver tissue to amplify a fragment of each muscle-specific gene. Each fragment was cloned using Invitrogen's TOPO-TA cloning kit, then sequenced to determine orientation of insertion. Standards were generated by in vitro transcription of each clone using Promega's Riboprobe in vitro Transcription System (Madison, WI). Transcripts were run on formaldehyde/MOPS gels to confirm the presence of a single band of the correct size, then quantified using a spectrophotometer. The molecular weight of the in vitro transcribed RNA was calculated using the following formula: MW = (# of A bases × 328.2) + (# of U bases × 305.2) + (# of C bases × 304.2) + (# of G bases × 344.2) + 159. Using the MW and concentration of each sample, copy #/μL was determined. These transcripts were used as quantitative standards to determine absolute mRNA copy number in each experimental sample. In addition, as a cellular mRNA control, *β-actin* levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999).

2.4. Data analysis

The data are reported as a ratio of absolute mRNA copy number of each muscle-specific gene to the absolute mRNA copy number of *β-actin*, multiplied by a constant for ease of interpretation, and expressed as means ± standard errors. Microsoft Excel was used to produce graphical representations of the data. Statistical analysis of the data was performed using Statistica6 (StatSoft) software. ANOVA was used with Newman–Keuls for comparisons between time point and level of gene expression. Significance levels for all comparisons were set at $P<0.05$.

3. Results

3.1. Metabolic response to starvation and refeeding

The expression levels of several genes indicative of specific metabolic pathways were tested for their response to starvation and refeeding. After 30 days of starvation, the level of *phosphoglycerate kinase* dropped significantly (Fig. 1A), and expression was reduced further after 14 days of refeeding (Fig. 1A). The expression level of *pyruvate kinase* was significantly increased after 30 days of starvation, then returned to original levels during refeeding (Fig. 1B). After a 30-day starve, *phosphoglucomutase* expression fell significantly and remained low during refeeding (Fig. 1C). The expression level of *fructose 1,6-bisphosphatase* was significantly increased after 30 days of starvation (Fig. 1D). This dramatic increase was followed by a dramatic decrease after 4 days of refeeding, then a significant increase after 14 days of refeeding (Fig. 1D).

Expression levels of *transaldolase* did not change during starvation, but rose significantly after four or 14 days of refeeding (Fig. 1E). Levels of *AldolaseB* expression did not change after 30 days of starvation (Fig. 1F). After 4 days of refeeding, a significant increase in expression was detected, and by 14 days of refeeding, a dramatic increase was detected (Fig. 1F).

3.2. Muscle response to starvation and refeeding

The primary MRF *TMyoD2* showed no significant change in expression in response to starvation and refeeding (Fig. 2A).

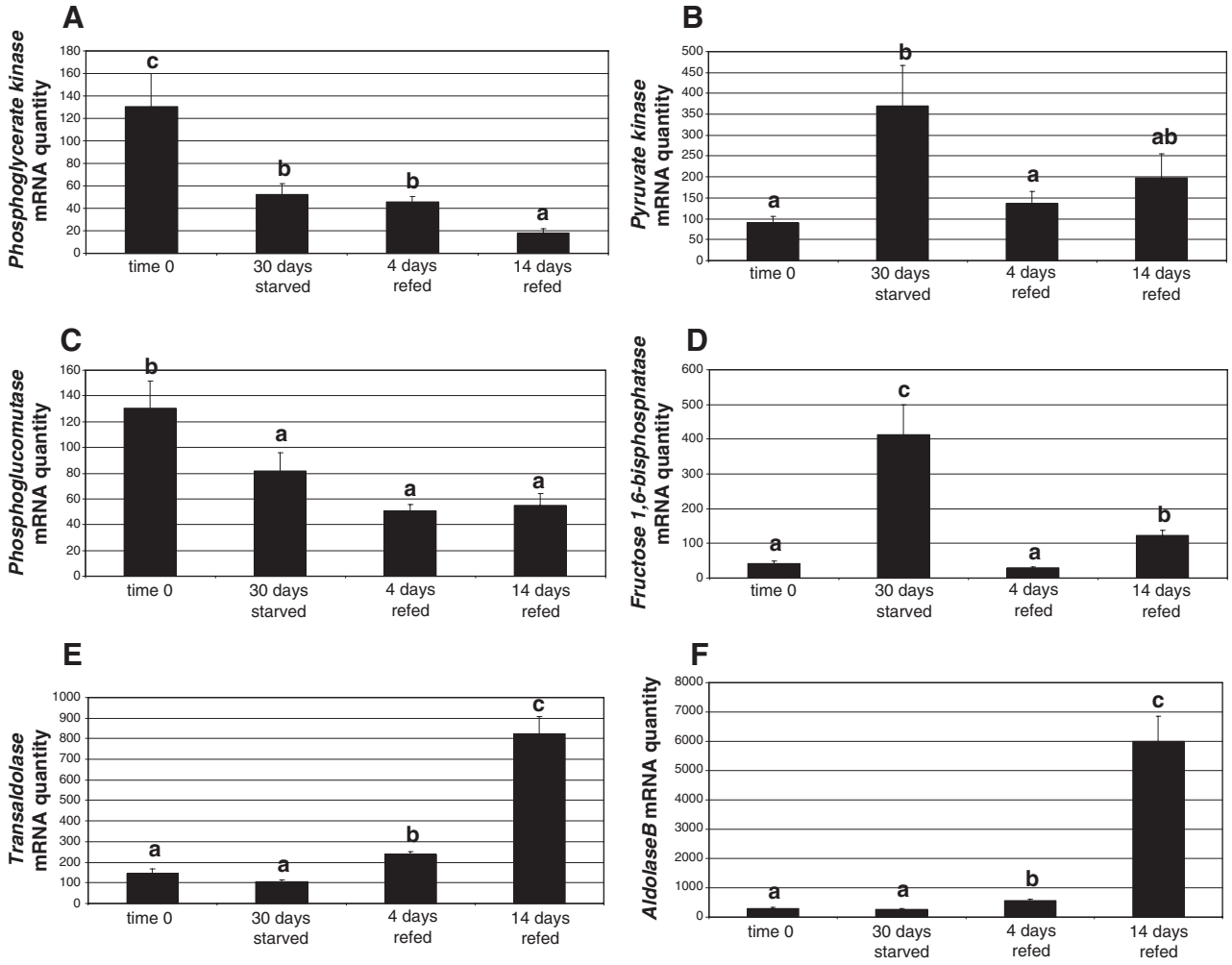

Fig. 1. Expression levels of metabolism genes. Mean ± S.E. of each mRNA quantity is shown for each time point tested. Quantities were determined by dividing the absolute copy number by the copy number of β-actin in each sample, then multiplying by a common factor. Quantities that are significantly different at $P<0.05$ are labeled with different letters.

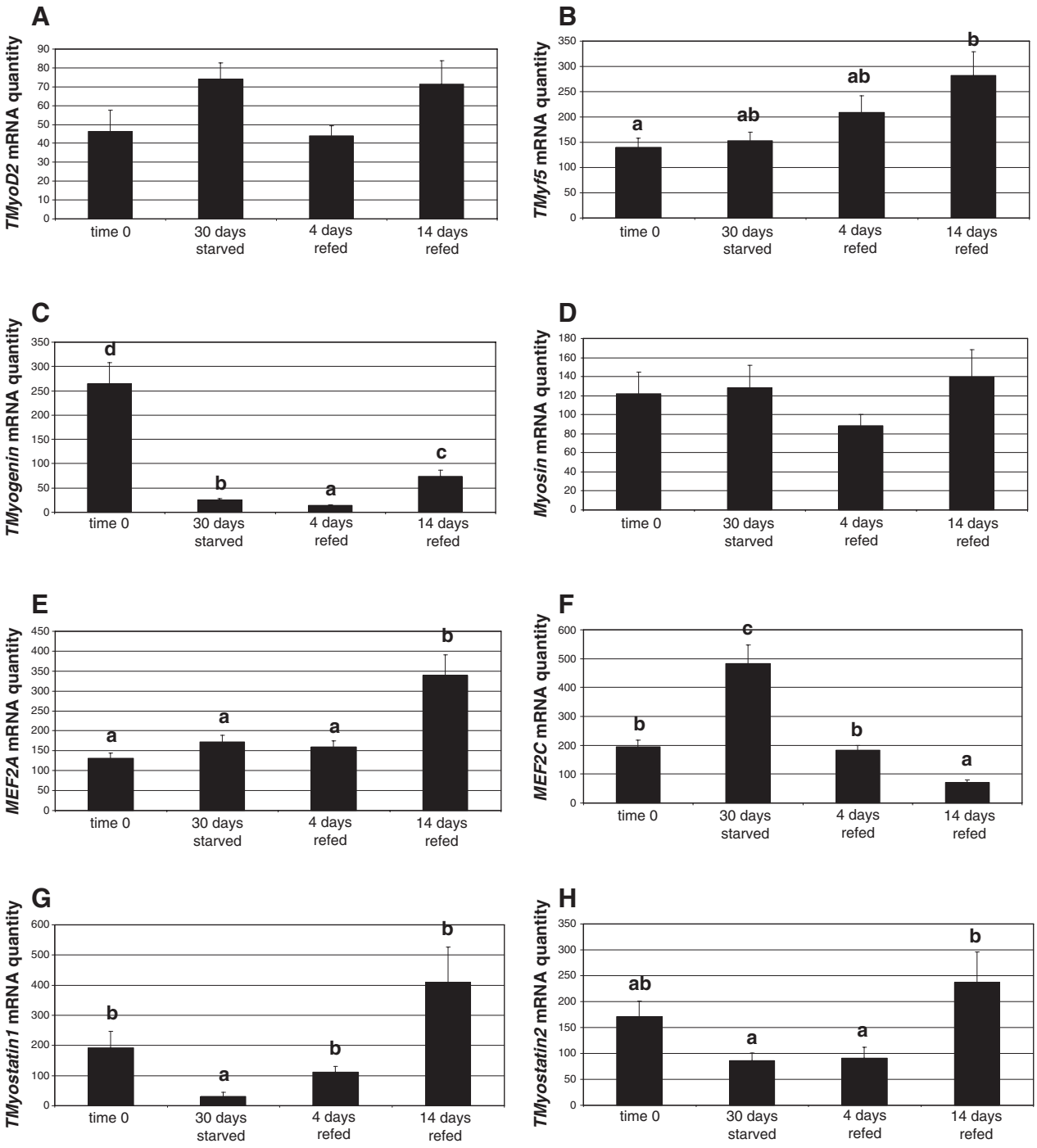

Fig. 2. Expression levels of muscle-specific genes. Mean ± S.E. of each mRNA quantity is shown for each time point tested. Quantities were determined by dividing the absolute copy number by the copy number of β-actin in each sample, then multiplying by a common factor. Quantities that are significantly different at $P<0.05$ are labeled with different letters.

Another primary MRF, *TMyf5*, also showed very few changes in expression in response to starvation and refeeding. The only significant increase in expression is found when levels after 14 days of refeeding are compared to those before the starvation (Fig. 2B).

The secondary MRF *Tmyogenin* shows a dramatic response to starvation and refeeding. After 30 days of starvation, *Tmyogenin* expression drops significantly (Fig. 2C). Expression continued to decrease after 4 days of refeeding, but rose significantly after 14 days of refeeding (Fig. 2C). In contrast to *Tmyogenin*, *myosin* showed no response to starvation and refeeding (Fig. 2D).

MEF2A and *MEF2C* showed differing reponses to starvation and refeeding. Expression of *MEF2A* increased significantly after 14 days of refeeding (Fig. 2E). In contrast to *MEF2A* expression, *MEF2C* expression increased significantly after 30 days of starvation, and decreased after four and 14 days of refeeding (Fig. 2F).

The *myostatin*s showed similar responses to starvation and refeeding. *TMyostatin1* expression decreased after 30 days of starvation, and increased significantly after 4 days of refeeding (Fig. 2G). Expression of *Tmyostatin2* decreased slightly but insignificantly after 30 days of starvation and 4 days of refeeding, then increased significantly after 14 days of refeeding (Fig. 2H).

4. Discussion

Metabolic alterations during starvation and refeeding can be extrapolated by analyzing expression levels of certain genes. Although some of the tested genes encode enzymes with reversible activity, nutritional state at the time of testing determines the most likely direction of the pathway and activity of the reversible enzyme. Metabolic state has a significant effect on muscle growth, and it is likely that changes in expression of muscle-specific genes are the result of changes in metabolic state (Hornick et al., 2000). Analysis of the changes in expression of muscle-specific genes during starvation and refeeding can be used to infer growth characteristics of muscle during these periods.

4.1. Metabolism and muscle growth during starvation

During starvation, metabolism shifts so that pathways designed to derive energy from storage sources are activated, while the pathways designed to derive energy from ingestion of food sources are downregulated (Salway, 2004). Analysis of higher vertebrate systems shows that muscle tissue shifts almost entirely away from glucose for fuel during this time (Stryer, 1995). Thus, glycolysis, the processing of glucose to generate ATP, the pentose phosphate pathway, a mechanism of generating NADPH and ribose-5-phosphate for nucleotide and nucleic acid synthesis, and fatty acid synthesis, are down-regulated. Glycogenolysis, which uses reserves of glycogen for entry into glycolysis, and gluconeogenesis, the production of glucose from glycerol, lactate, and amino acids, are upregulated (Salway, 2004; Stryer, 1995). The decrease in expression of *phosphoglycerate kinase* and *transaldolase*, the static levels of *aldolaseB*, and the increase in expression of *fructose 1,6-bisphosphatase* reflect these shifts in metabolism. Although the reduction in the level of *phosphoglucomutase* after 30 days of starvation suggests a reduction in glycogenolysis, it is likely that glycogenolysis was only active during the beginning of the starvation period. In most animals, glycogen reserves are exhausted quickly during starvation (<24 h in humans; Stipanuk, 2000), after which time *phosphoglucomutase* levels most likely decline.

It is somewhat unexpected that expression of *pyruvate kinase*, another marker of glycolysis, does not decrease during starvation. In fact, *pyruvate kinase* expression increases (Fig. 1B). This expression may be indicative of another function of pyruvate kinase, its requirement in the breakdown of muscle protein to generate fuel during starvation. Pyruvate kinase is required to catabolize specific amino acids generated during the breakdown of muscle protein, which can then be used for gluconeogenesis and entry into the Krebs cycle (Salway, 2004). Pyruvate kinase exists in several different tissue-specific isoforms, each regulated in different ways (Muñoz and Ponce, 2003). The nucleotide sequences of the liver and muscle isoforms are similar (Yamada and Noguchi, 1999; Genbank), and although the tissue in which expression was tested in this study was muscle, it is possible that this expression represents either the liver-specific isoform, or both liver and muscle. The liver specific isoform is activated by fructose-1-bisphosphate (Muñoz and Ponce, 2003), so it is also possible that expression of *pyruvate kinase* increases because of an abundance of fructose 1,6-bisphosphate generated during gluconeogenesis.

The major site of gluconeogenesis is the liver, and it is thought that very little gluconeogenesis happens in the muscle (Salway, 2004). However, the fact that *fructose 1,6-bisphosphatase* expression is responsive to nutritional status argues that gluconeogenesis does take place in the muscle. Similar to this study, Byrne et al. (2005) found evidence that caloric restriction may increase metabolism of amino acids via gluconeogenesis. Panserat et al. (2001) found the opposite, i.e., no *fructose 1,6-bisphosphatase* expression was detected in the muscle of fish that had been starved for 4 days. It is possible that a 4-day fast was too short to elicit a major change in *fructose 1,6-bisphosphatase* expression, or that the methods of Panserat et al. (2001) were not sensitive enough to detect expression.

Since very little muscle growth is normally observed during starvation, significant down-regulation of genes that promote muscle growth was expected. Surprisingly, there were few changes in the expression of *TMyoD2*, *TMyf5*, *MEF2A*, and *myosin*. However, the expression of *Tmyogenin* fell dramatically after a 30-day starvation (Fig. 2C), suggesting a major reduction in the amount of myotube hypertrophy occurring during starvation. Myogenin levels appear to be correlated with nutritional state in other organisms as well. Expression significantly decreased after 1 week of reduced rationing in sheep, and remained low through the 22-week reduced rationing period (Jeanplong et al., 2003).

Unexpectedly, *MEF2C* expression rose after 30 days of starvation (Fig. 2F). It is possible that the rise is a response to the apparent reduction in myotube hypertrophy/maturation that begins to occur during starvation in an attempt to lessen the severity of the muscle loss. This pattern of *MEF2C* expression has previously been observed in spawning adults, whose muscle is wasting as a result of decreased nutrient intake and increased energy utilization required for spawning behaviors. In spawning adults, *MEF2C* expression increased, possibly as a mechanism to combat muscle atrophy during the spawning season (Johansen and Overturf, 2005b).

It is tempting to assume that expression of *TMyostatin1* and *TMyostatin2*, genes that negatively regulate muscle growth, may be upregulated during times of slow growth, e.g., starvation periods. However, our results demonstrate the opposite; both *Tmyostatin1* and *Tmyostatin2* expression levels fell after 30 days of starvation (Fig. 2G, H). According to these results, it seems unlikely that decreased muscle growth in starved fish is due to the increased expression of the *myostatin*s.

Instead, downregulation of *myostatin* expression may be a mechanism of lessening the muscle atrophy occurring during starvation. Consistent with this notion, previous experiments in spawning adults exhibiting muscle atrophy have shown that the *myostatin*s are unlikely to be the cause of the atrophy (Johansen and Overturf, 2005b). Similarly, tilapia subjected to a long-term fast showed decreased body weight but no change in *myostatin* levels (Rodgers et al., 2003).

Taking the expression levels of the metabolic and muscle-specific factors together, it appears that during starvation, the reductions in glycolysis, glycogenesis, and pentose phosphate activity, and the increase in gluconeogenesis initiate changes in muscle-specific genes that result mainly in the reduction of myotube hypertrophy.

4.2. Metabolism and muscle growth during refeeding

During periods of satiate feeding, excess nutrients enter the system, triggering pathways designed both to metabolize the nutrients for energy and to convert it to storage forms for use during periods of less abundant food availability (Salway, 2004). For example, increases in expression of genes active in pathways such as glycolysis, glycogenesis, and fatty acid synthesis, and decreases in expression of genes active in pathways such as gluconeogenesis may be expected. However, this period of satiate feeding was preceded by a 30-day starve. It is possible that metabolic activities are altered in such situations, and are accompanied by an increased growth plane (Hornick et al., 2000). The expression levels of several genes controlling metabolism and muscle growth were tested to determine whether changes in these pathways could be observed.

Many species of fish metabolize glucose poorly (Moon, 2001), and the failure to upregulate factors such as *pyruvate kinase* and *phosphoglycerate kinase* is likely an indication that glycolysis is not active. However, in aquaculture, carbohydrates make up over 20% of the diet (Hardy, 2002), and this influx of glucose must be processed in some fashion. Instead of glycolysis, glucose may enter the pentose phosphate pathway to either generate energy in the form of NADPH to drive fatty acid synthesis or to produce ribose-5-phosphate for incorporation into nucleotides and nucleic acids (Salway, 2004). Expression of *transaldolase* increased during the refeeding period, suggesting involvement of the pentose phosphate pathway in processing the consumed feed. The majority of fatty acid synthesis takes place in the liver, not the muscle, the tissue from which these expression levels were observed. It therefore appears that, in addition to producing NADPH to drive fatty acid synthesis, the pentose phosphate pathway in the muscle of refeeding fish also produces the nucleotides and nucleic acids that are essential for the DNA and RNA synthesis that occurs during times of rapid growth.

Glycogenic activity is often upregulated during satiate feeding periods as glucose is converted into its storage form, glycogen (Stryer, 1995). The upregulation of *phosphoglucomutase* expression would exemplify this activity. However, *phosphoglucomutase* was not upregulated during refeeding (Fig. 1C). Glycogenesis happens very quickly after feeding, so it is likely that any increase in glycogenic activity has already happened by 4 days after refeeding. Alternatively, given the increase in *transaldolase* expression (Fig. 1E), glucose may be preferentially used for entry into the pentose phosphate pathway instead of being used for glycogenesis.

Gluconeogenic activity fell dramatically when refeeding was initiated, as indicated by the significant decrease in the expression of *fructose 1,6-bisphosphatase* after 4 days of refeeding (Fig. 1D). This is not a surprising result given that gluconeogenesis is normally not as active during times of satiate feeding. However, after 14 days of refeeding, *fructose 1,6-bisphosphatase* expression increased slightly, possibly because of the increased activity of the pentose phosphate pathway, which requires fructose 6-phosphate, a product of fructose 1,6-bisphosphatase activity.

Expression of *aldolaseB* was increased in fish refeeding after starvation (Fig. 1F). During the refeeding period, large amounts of glucose were ingested, and in turn, insulin was most likely secreted (Mommsen and Plisetskaya, 1991). In rats, insulin and glucose stimulate transcription of *aldolaseB* (Decaux et al., 1991), suggesting that these factors may also stimulate expression in trout. AldolaseB activity generates dihydroxyacetone phosphate and glyceraldehyde, which can both be converted to glyceraldehyde 3-phosphate and enter glycolysis. However, expression of genes encoding enzymes active in glycolysis (*pyruvate kinase* and *phosphoglycerate kinase*) was not upregulated during refeeding, suggesting a different fate for the dihydroxyacetone phosphate and glyceraldehyde generated. Instead of entering glycolysis, dihydroxyacetone phosphate and glyceraldehyde may be converted to glycerol 3-phosphate, which can be esterified to form tripalmitin, a fatty acid. Expression of *aldolaseB* is approximately 10 times higher during the refeeding period than in the period before the starvation began, implying that there is more fatty acid formation during refeeding periods than during normal feeding. This may represent an alteration in metabolism that partially accounts for the increased growth plane often observed during refeeding periods (Hayward et al., 1997).

Similar to what was seen during the starvation period, the expression levels of *TMyoD2* and *TMyf5* showed little change during the refeeding period (Fig. 2A, B). These results suggest that myoblast specification and proliferation are not induced at a higher rate by *TMyoD2* or *TMyf5* during refeeding. It is therefore unlikely that the basis of the rapid growth rate observed in refeeding fish is due to an increase in myoblast specification and proliferation.

Expression of *Tmyogenin* and *MEF2A* is more responsive to refeeding than expression of *TMyoD2*, *Myf5*, or *myosin* (Fig. 2C, E). In the case of *Tmyogenin*, expression during refeeding is approximately one-third of the amount seen prior to the starvation period (Fig. 2C), but nonetheless, expression of it and *MEF2A* suggests that muscle growth by myotube hypertrophy is occurring by 14 days after the refeeding begins. Since both TMyogenin and MEF2A signal through several intermediates to activate transcription of *myosin* (Watabe, 2001; Molkentin and Olson, 1996), it is unexpected that an increase in *myosin* expression was not detected. It is possible that

Tmyogenin and/or *MEF2A* expression must be at a higher level to significantly increase *myosin* expression, or that the response of *myosin* expression to an upregulation of Tmyogenin and/or MEF2A takes longer than what has been captured in the 14-day refeeding period. While conditions that alter muscle growth, including temperature change, usually affect myosin expression, it has been observed that a reduction in temperature had no effect on the expression of myosin at hatching of rainbow trout fry (Xie et al., 2001). It is also possible that a constant level of *myosin* expression is maintained in fish of this size and only minimal changes in translation are necessary to supply new muscle. In support of this thought, Svanberg et al. (2000) found that postprandial regulation of myosin occurred mainly at the translational level.

Expression of *MEF2C* shows the opposite response of *Tmyogenin* and *MEF2A*, exhibiting significant decreases after four and 14 days of refeeding (Fig. 2F). Although this pattern of expression is unexpected for a factor thought to promote muscle growth, it is consistent with the notion that *MEF2C* expression is triggered by a loss in muscle mass as a mechanism of fighting the loss. Expression would therefore fall during periods of satiate feeding and concomitant muscle growth. This implies differential regulation of *MEF2A* and *MEF2C*, with *MEF2A* being either stimulated during satiate feeding or inhibited during starvation, and *MEF2C* being either stimulated during starvation or inhibited during satiate feeding.

The *myostatin*s show similar responses to refeeding. *Tmyostatin1* expression increases significantly after 4 days of refeeding (Fig. 2G), and *Tmyostatin2* expression increases significantly after 14 days of refeeding (Fig. 2H). Since the myostatins are thought to negatively regulate muscle growth, these expression patterns, taken together, indicate inhibition of muscle growth during the refeeding period. However, during refeeding periods, an increase in growth rate is usually observed. Although this does not directly indicate that muscle is actively growing, it implies that muscle growth is not inhibited. The increased *myostatin* expression observed during the refeeding period, instead of inhibiting muscle growth, may more likely function to attenuate the muscle growth that is occurring as a result of the increased expression of pro-muscle growth factors, such as *Tmyogenin*.

Feed restriction and refeeding induced changes in metabolism that affected the molecular control of muscle growth. The genes that control myotube hypertrophy and maturation appeared to be most affected by metabolic alterations. These results demonstrate the importance to aquaculture of understanding the effect of alternative feeding programs on muscle structure, and in turn, fillet quality.

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